

# Troglitazone Inhibits Cyclin D1 Expression and Cell Cycling Independently of PPAR $\gamma$ in Normal Mouse Skin Keratinocytes

Guobin He,\* Philippe Thuillier,† and Susan M. Fischer\*

\*The University of Texas M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, Texas, USA; †Department of Nutrition, University of North Carolina, Chapel Hill, North Carolina, USA

Troglitazone is one of the thiazolidinedione (TZD) class of anti-diabetic drugs and a ligand for peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ). Troglitazone and other PPAR $\gamma$  ligands have been shown to inhibit cell proliferation and induce cell cycle arrest in a variety of cancer cells, and have been considered as potential tumor preventive and tumor therapeutic agents. Little is known, however, about how normal or initiated cells respond to these agents during mouse skin carcinogenesis. We report here that troglitazone and another TZD, ciglitazone, dramatically inhibited mitogen-induced cellular proliferation in normal mouse skin primary keratinocytes and in the C50 keratinocyte cell line. This was accompanied by induction of cell cycle G1 phase arrest and suppression of cyclin D1, cdk4, and cdk2 expression. Troglitazone suppressed cyclin D1 expression at multiple levels. In addition, we demonstrated that PPAR $\gamma$  was not expressed at functional levels in cultured mouse skin keratinocytes, and that the inhibitory effects of troglitazone on cellular proliferation and cyclin D1 expression in these cells were PPAR $\gamma$ -independent. Given the important role of keratinocyte proliferation in skin carcinogenesis, our data suggest that TZD may be useful tumor preventive agents in skin.

Key words: nuclear receptors/proliferation/thiazolidinediones  
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Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors that belong to the nuclear receptor superfamily (Issemann and Green, 1990). Three isoforms, namely, PPAR $\alpha$ , PPAR $\delta$ , and PPAR $\gamma$ , have been identified so far and PPAR $\gamma$  is one of the most intensively studied PPAR isoforms. Ligand-activated PPAR $\gamma$  transactivates genes by heterodimerizing with retinoid X receptor and binding to a consensus DNA sequence called PPAR response element (PPRE) in the promoter region of target genes. PPAR $\gamma$  plays a key role in adipogenesis (Barak *et al*, 1999). PPAR $\gamma$  knockout mice are embryonic lethal and are completely devoid of all types of adipose tissue (Barak *et al*, 1999). Consistent with a role of PPAR $\gamma$  in lipid metabolism, several metabolites of arachidonic acid have been found to be selective PPAR $\gamma$  ligands with high affinity. These include 15-deoxy- $\Delta$ 12,14-prostaglandin J<sub>2</sub> (15-PGJ<sub>2</sub>), 13-hydroxyoctadecadienoic acid and 15-hydroxy-eicosatetraenoic acid (reviewed in Rosen and Spiegelman, 2001). Interestingly, thiazolidinediones (TZD), a novel class of synthetic anti-diabetic drugs, were also identified as selective PPAR $\gamma$  ligands (Lehmann *et al*, 1995).

Studies using PPAR $\gamma$  ligands have implicated PPAR $\gamma$  in many diverse pathways including control of cellular proliferation and differentiation. For instance, ligand activation of endogenous or ectopically expressed PPAR $\gamma$  is sufficient to induce growth arrest in fibroblasts and preadipocytes and leads to formation of adipocytes (Altioek *et al*, 1997). The growth arrest is due to a decrease in DNA binding and transcriptional activity of the E2F/DP complex. This observation has led to successful efforts in using PPAR $\gamma$  ligands to induce growth arrest and terminal differentiation in cultured human liposarcoma cells (Tontonoz *et al*, 1997). PPAR $\gamma$  ligands were later found to have the capacity to inhibit cell growth in a wide variety of tumor cells not necessarily of adipose lineage (reviewed in Fajas *et al*, 2001). Notably, application of troglitazone, a TZD drug, leads to cell growth arrest and induces expression of differentiation markers in a number of colon cancer cell lines (Sarraf *et al*, 1998). Tumors derived from human colon cancer cells grown in nude mice were 50% smaller when mice were fed troglitazone (Sarraf *et al*, 1998). Similarly, troglitazone inhibits clonal growth of MCF-7 breast cancer cells. The retinoic acid receptor ligand all-*trans*-retinoic acid synergizes the inhibitory effect of troglitazone and irreversibly leads to apoptosis of MCF-7 cells (Elstner *et al*, 1998). Normal human prostate expresses very low levels of PPAR $\gamma$  and PPAR $\gamma$  levels are highly elevated in prostate tumors. Culture of surgically obtained human prostate cancer tumors with troglitazone produces marked and selective necrosis of the cancer cells (about 60%) but not the

Abbreviations: dnPPAR $\gamma$ , dominant negative PPAR $\gamma$ ; Erk, extracellular signal-regulated kinase; GFP, green fluorescent protein; KGM-2, keratinocyte growth medium-2; 15-PGJ<sub>2</sub>, 15-deoxy- $\Delta$ 12, 14-prostaglandin J<sub>2</sub>; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; PPRE, PPAR response element; SDS, sodium dodecyl sulfate; TZD, thiazolidinediones

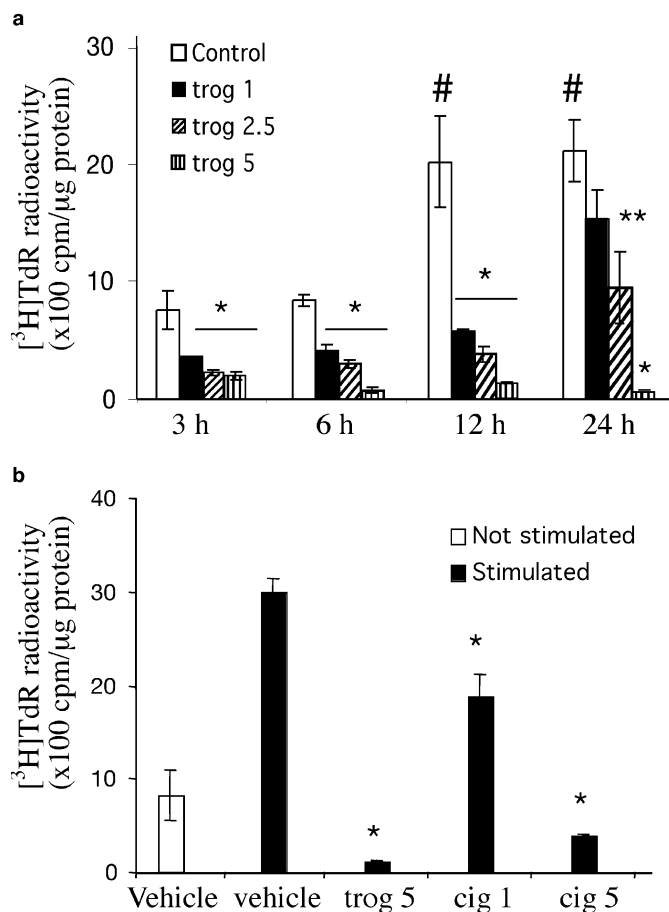
adjacent normal prostate cells (Kubota *et al*, 1998). These findings have raised considerable interest in the possible use of PPAR $\gamma$  ligands as tumor-preventive and tumor-therapeutic agents.

Although most studies focus on the anti-growth effect of PPAR $\gamma$  ligands on tumor cells, very little is known about how normal cells respond to these ligands during carcinogenesis. We show here that TZD dramatically inhibited mitogen-induced cellular proliferation and caused a G1 phase arrest in normal mouse skin keratinocytes. The cell cycle arrest by troglitazone was accompanied by suppression of cyclin D1 expression at multiple levels. Unexpectedly, we found that PPAR $\gamma$  was not expressed at functional levels in cultured keratinocytes. Our data indicate that troglitazone inhibits cyclin D1 expression and mouse skin keratinocyte proliferation through PPAR $\gamma$ -independent mechanisms.

## Results

**Inhibition of cellular proliferation and cell cycle progression of keratinocytes by troglitazone** To test whether TZD have the potential to be used as tumor-preventive agents in mouse skin, we determined whether TZD could affect the proliferative response of normal keratinocytes to mitogen stimulus. We cultured primary keratinocytes and C50 cells in serum-free medium (KGM-2 (keratinocyte growth medium-2)) for 2 d without changing medium. Cells were then stimulated with fresh KGM-2 medium and treated with the PPAR $\gamma$  ligand troglitazone. KGM-2 medium contains epidermal growth factor and insulin and other components that are required for growth of keratinocytes. [ $^3$ H]thymidine incorporation assay reflects cellular DNA synthesis activity and is frequently used as a marker of cellular proliferation. As shown in Fig 1a, application of fresh KGM-2 medium stimulated DNA synthesis activity in primary keratinocytes at 12 and 24 h and troglitazone suppressed this induction in a dose-dependent manner. Five  $\mu$ M troglitazone inhibited DNA synthesis by 97.2% ( $p < 0.01$ ) at 24 h. The increased DNA synthesis activity at 12 and 24 h in the control cells is likely the result of quiescent cells re-entering the cell cycle after stimulation by fresh medium. DNA synthesis in the control cells was almost unchanged at 6 h compared with 3 h, suggesting that it took more than 6 h for these quiescent cells to re-enter the cell cycle and finally reach S phase. The inhibitory effect of troglitazone on DNA synthesis, however, was already evident as early as 3 h. We interpret this to mean that troglitazone inhibits keratinocyte proliferation by inhibiting the progression of cycling cells into S phase at least at early time points. We do not know from these data whether troglitazone also inhibits the re-entry of quiescent cells into cell cycle. Troglitazone inhibited DNA synthesis of C50 cells in a similar pattern (data not shown and Fig 1b). Another TZD compound ciglitazone also inhibited C50 cell proliferation in a concentration-dependent manner, although it is not as potent as troglitazone (Fig 1b).

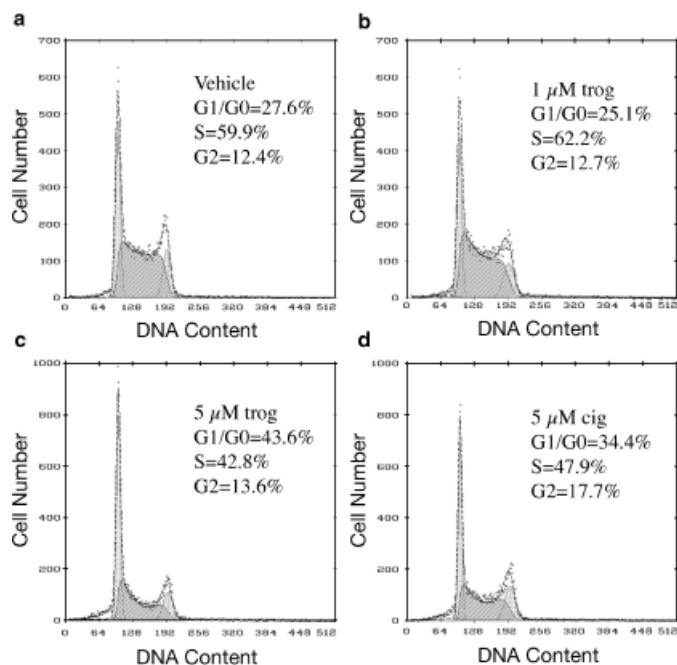
To gain insight as to what phases of the cell cycle are affected by troglitazone, C50 cells were treated with 1 or 5  $\mu$ M troglitazone for 24 h and flow cytometry for DNA content analysis was performed. Although 1  $\mu$ M troglitazone



**Figure 1**  
**Inhibitory effect of thiazolidinediones (TZD) on DNA synthesis of keratinocytes.** (a) Mouse skin primary keratinocytes at 80% confluence were stimulated with fresh keratinocyte growth medium-2 (KGM-2) medium and treated with vehicle (control) or 1–5  $\mu$ M troglitazone (Trog 1–5) for the indicated periods of time. (b) C50 cells at 80% confluence were not stimulated or stimulated with fresh KGM-2 medium and treated for 24 h with vehicle, 5  $\mu$ M Trogl (Trog 5), 1 or 5  $\mu$ M ciglitazone (cig 1 or cig 5, respectively). Cells were pulsed with 1  $\mu$ Ci per mL of [ $^3$ H]thymidine for 2 h before harvest. The [ $^3$ H]thymidine ([ $^3$ H]Tdr) incorporated by cells was measured and normalized to protein concentration. Cells were treated in triplicate. A set of representative data from three independent experiments is presented and values are presented as mean  $\pm$  SD. \* $p < 0.01$ ; \*\* $p < 0.05$  versus vehicle control of similar conditions; #  $p < 0.05$  versus vehicle control at 3 or 6 h.

showed no effect on cell cycle of C50 cells, 5  $\mu$ M troglitazone induced a strong accumulation of cells in G1 phase (Fig 2b and c). These data provide evidence that troglitazone inhibits the transition of cells from G1 phase to S phase. Five  $\mu$ M ciglitazone induced a similar G1 phase arrest. Interestingly, it also slightly increased G2 phase population in C50 cells (Fig 2d).

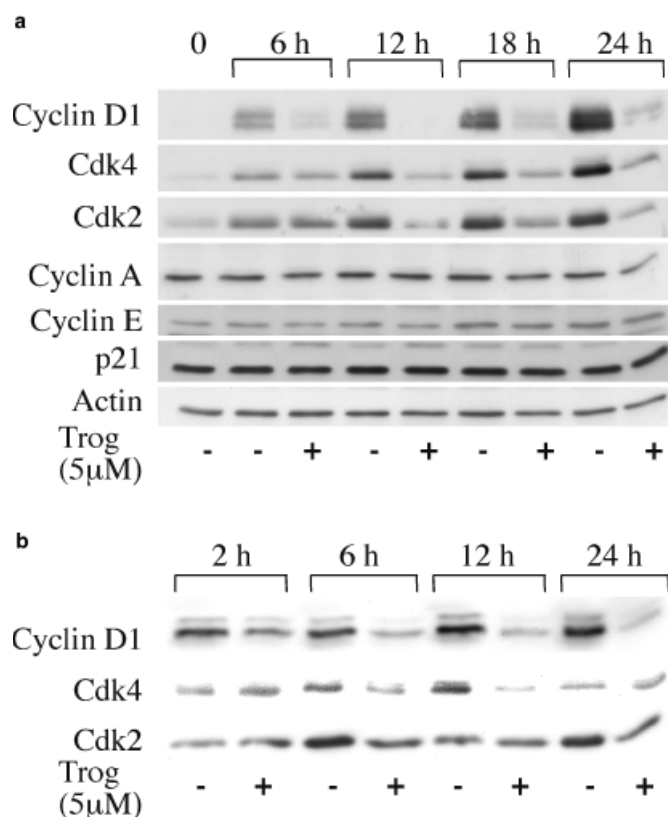
**Suppression of cyclin D1 expression by troglitazone** To investigate the mechanisms responsible for the G1 arrest by troglitazone, we examined its effect on the expression of cell cycle proteins by western blot analysis. Change of medium at the beginning of treatment caused increased protein expression of cyclin D1, cdk2, and cdk4 in the control group. Cyclin D1 and cdk2/cdk4 protein levels, however, remained low for up to 24 h when C50 cells were



**Figure 2**  
**Cell cycle arrest induced by troglitazone (Trog).** C50 cells at 80% confluence were stimulated with fresh keratinocyte growth medium-2 (KGM-2) medium and treated for 24 h with (a) vehicle, (b) 1  $\mu$ M Trog (Trog), (c) 5  $\mu$ M Trog (Trog), (d) 5  $\mu$ M ciglitazone (cig). Propidium iodide-stained single-cell suspensions were obtained and DNA content analyses were performed. A set of representative data from two independent experiments is presented.

treated with 5  $\mu$ M troglitazone (Fig 3a). We did not see significant changes in other cyclins or the cdk inhibitor p21 up to 24 h after troglitazone treatment (Fig 3a). Troglitazone inhibited expression of cyclin D1, cdk2/cdk4 in a similar pattern in primary keratinocytes as in C50 cells (Fig 3b). Inhibition of cyclin D1 was evident as early as 2 h (Fig 3b), although changes in cdk2/cdk4 levels only occurred after 6 h. These findings suggest that cyclin D1 may be an important target for troglitazone in terms of cell cycle arrest, especially with respect to the G1 phase arrest.

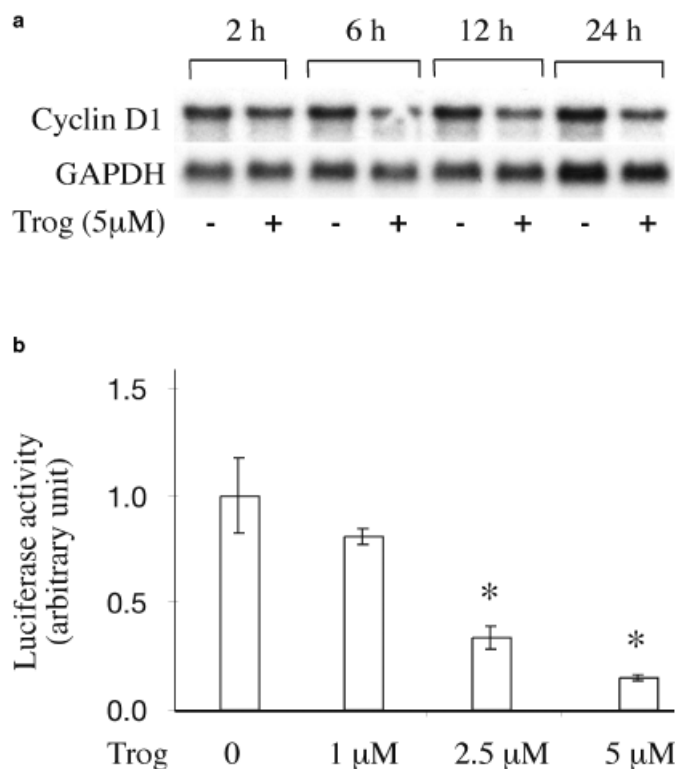
Next, we were interested in determining how cyclin D1 was regulated by troglitazone. As shown in Fig 4a, 5  $\mu$ M troglitazone suppressed mitogen-induced cyclin D1 mRNA expression in C50 cells, suggesting that troglitazone may inhibit cyclin D1 expression at the transcriptional level. We tried to determine the effect of troglitazone on cyclin D1 mRNA stability using actinomycin D, but we were not successful because actinomycin D appeared to stabilize existing cyclin D1 mRNA (data not shown), as has been reported by others (Lin *et al*, 2000). To further show that troglitazone inhibits cyclin D1 expression at the transcriptional level, primary keratinocytes were transiently transfected with a luciferase reporter construct under the control of a 1745 bp cyclin D1 promoter. Treatment of various concentrations of troglitazone inhibited cyclin D1 promoter activity in a concentration-dependent manner (Fig 4b). Similar results were obtained in C50 cells (data not shown). Since the inhibitory effect of troglitazone on cyclin D1 expression at the protein level appears stronger than at the mRNA level, we suspected that regulation other than at the level of transcription may also be involved. Cyclin D1 protein



**Figure 3**  
**Suppression of mitogen-induced cell cycle protein expression by troglitazone (Trog).** C50 cells (a) or primary keratinocytes (b) were stimulated with fresh keratinocyte growth medium-2 (KGM-2) medium and treated with 5  $\mu$ M Trog (Trog) for the indicated periods of time. Attached cells were harvested in radioimmunoprotein assay buffer. Thirty micrograms protein from whole-cell extracts was used for western blot analysis and immunostained with antibodies against indicated proteins. A set of representative data from at least two independent experiments is presented.

degradation is a possible target for regulation by troglitazone (Qin *et al*, 2003). We blocked proteasome activity by using proteasome inhibitors prior to troglitazone treatment and found that the proteasome inhibitors Alln and MG-132 partially inhibited the effect of troglitazone on cyclin D1 protein expression in C50 cells (Fig 5a and b). Troglitazone alone reduced cyclin D1 protein levels by 53%–55% of control levels, but only by 15%–18% of control levels in the presence of Alln or 10  $\mu$ M MG-132 (Fig 5a and b). We further examined the effect of troglitazone on the ubiquitination of cyclin D1 protein by immunoprecipitation. Troglitazone increased cyclin D1-bound ubiquitin, a small polypeptide that targets proteins for degradation through proteasomes (Fig 5c). These results suggest that troglitazone reduces cyclin D1 expression at least partially by enhancing degradation of cyclin D1 protein through the proteasome pathway.

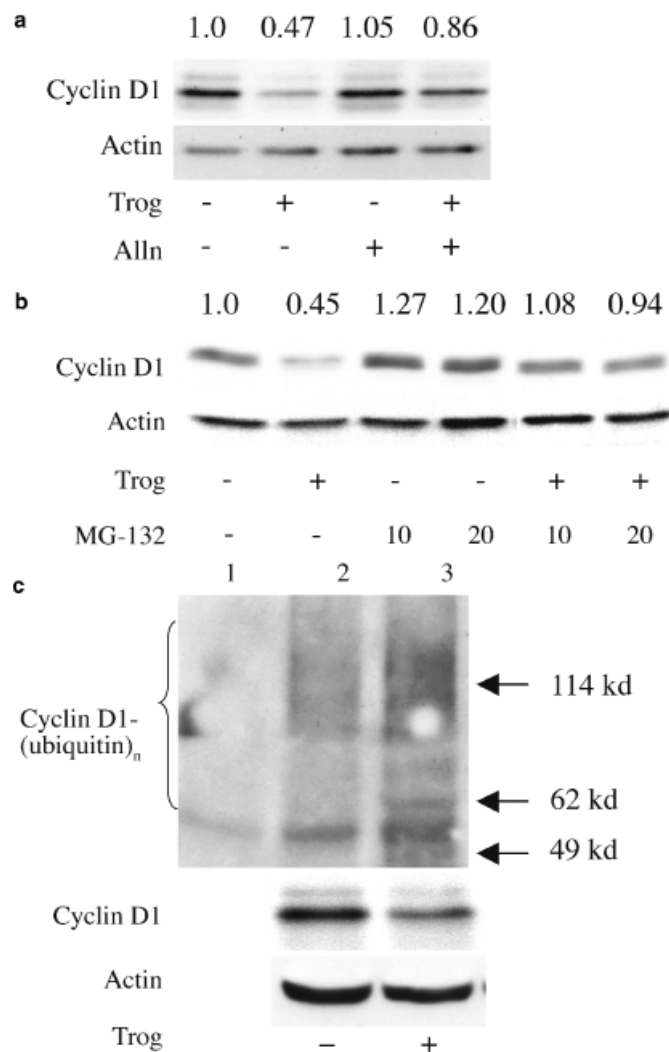
It has been shown that TZD drugs inhibit translation initiation in mouse embryonic stem cells (Palakurthi *et al*, 2001). To explore this possibility in keratinocytes, we measured the effect of troglitazone on incorporation of [ $^{35}$ S]methionine in C50 cells. As shown in Fig 6a, 5  $\mu$ M troglitazone inhibited total protein synthesis by 26% at 6 h. This may partially account for the inhibitory effect of troglitazone on cell proliferation and cyclin D1 expression



**Figure 4**  
**Suppression of cyclin D1 mRNA levels by troglitazone (Trog).** (a) C50 cells were stimulated with fresh keratinocyte growth medium (KGM-2) medium and treated with 5 μM Trog (Trog) for indicated periods of time. Cyclin D1 mRNA levels were determined by Northern blot analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (b) Primary keratinocytes in six-well plates were transiently transfected in triplicate with a luciferase reporter gene under the control of a cyclin D1 promoter and treated with vehicle or Trog (Trog 1–5 μM) for 24 h. Luciferase activity was measured and normalized to an internal control (β-galactosidase). Data are presented as mean ± SD. \**p* < 0.01 versus vehicle control. A set of representative data from at least three independent experiments is presented.

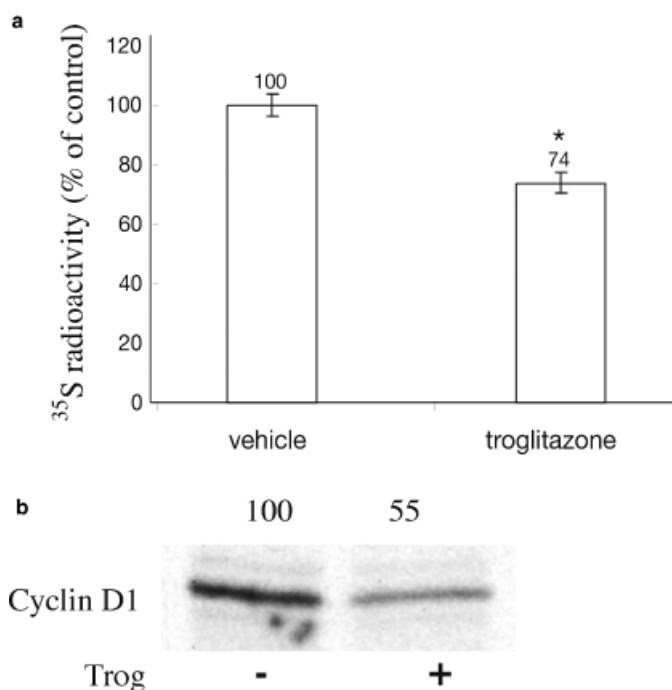
shown in Figs 1 and 3. Inhibition of cyclin D1 incorporation of [<sup>35</sup>S]methionine by troglitazone, however, was much stronger (45%) than of total protein incorporation (Fig 6b), which is consistent with the observation that troglitazone also inhibits cyclin D1 transcription and enhances cyclin D1 protein degradation.

**Lack of involvement of PPAR<sub>γ</sub> in troglitazone-induced growth inhibition** It has been clearly demonstrated that troglitazone and other TZD compounds are selective PPAR<sub>γ</sub> ligands, and PPAR<sub>γ</sub> is assumed to mediate most of their pharmacological action (Lehmann *et al*, 1995). As expected, troglitazone readily bound to ectopic PPAR<sub>γ</sub> in C50 cells as demonstrated with the Gal4-PPAR<sub>γ</sub>-LBD and MH-100-TK-LUC system (Nagy *et al*, 1998) (Fig 7a). As low as 0.2 μM troglitazone effectively bound to PPAR<sub>γ</sub> ligand binding domain and induced a 1.7-fold reporter expression, although PPAR<sub>α</sub> ligand (Wy-14643) did not. To test whether PPAR<sub>γ</sub> is involved in the anti-proliferative effect of troglitazone in keratinocytes, we examined whether a functional PPAR<sub>γ</sub> exists in keratinocytes. To do so, C50 cells were transiently transfected with a PPRE reporter construct. As shown in Fig 7b, troglitazone could not induce PPRE activity in C50 cells. Rather, it inhibited it. It is important to note



**Figure 5**  
**Enhanced cyclin D1 protein degradation by troglitazone (Trog).** C50 cells were pre-treated with specific proteasome inhibitor 200 μM Alln (a) 10 or 20 μM MG-132 (b) for 15 min. Cells were then treated with 5 μM Trog (Trog) for an additional 6 h. Thirty μg protein from whole-cell extracts was used for western blot analysis for cyclin D1 expression. β-actin (actin) was used as a loading control. Densitometric quantitation of the protein bands relative to actin expression is shown with the vehicle only samples set as 1.0. (c) C50 cells were treated with vehicle (lane 2), or 5 μM Trog (lane 3) for 6 h. 300 μg of whole-cell extract was immunoprecipitated with a cyclin D1 antibody and immunoblotted with an anti-ubiquitin antibody. The blot was then reprobed for cyclin D1 as an immunoprecipitation control. Lane 1 was an immunoprecipitation negative control (beads only). Lower panel: 1/10 of immunoprecipitation inputs were immunoblotted with anti-β-actin (actin) as a loading control. A set of representative data from at least two independent experiments is presented.

that troglitazone also inhibited CMV-driven β-galactosidase expression, an internal control used for transfection (Fig 7b, *inset*). This is probably due to the inhibitory effect of troglitazone on total protein synthesis. When a PPAR<sub>γ</sub> expression vector was co-transfected with the PPRE reporter construct, however, 1.8-fold induction of PPRE activity was observed (Fig 7b). Although the induction was modest, it was highly repeatable. The inhibitory effect of troglitazone on PPRE basal activity in the absence of exogenous PPAR<sub>γ</sub> may account for the modest induction. The modest induction of PPRE activity by troglitazone

**Figure 6**

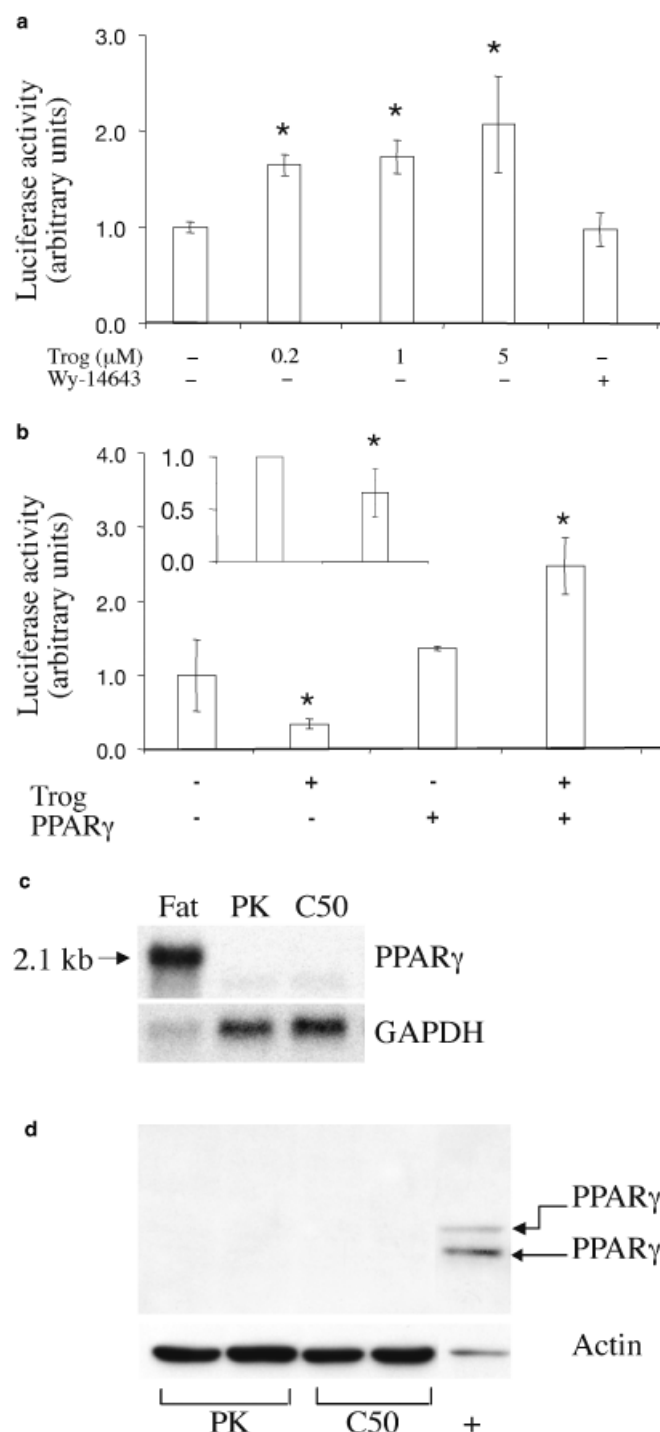
**Inhibition of protein synthesis by troglitazone (Trog).** C50 cells in triplicate were treated with vehicle (–) or 5  $\mu$ M Trog (+) for 5 h and pulsed with [<sup>35</sup>S]methionine for an additional 1 h. (a) [<sup>35</sup>S]Methionine incorporated by cells was measured. \* $p < 0.05$ , versus vehicle control. (b) [<sup>35</sup>S]methionine-labeled cyclin D1 was immunoprecipitated as described in the Materials and Methods. The bands were quantitated by densitometry and data are presented as % of control with vehicle control set to 100%.

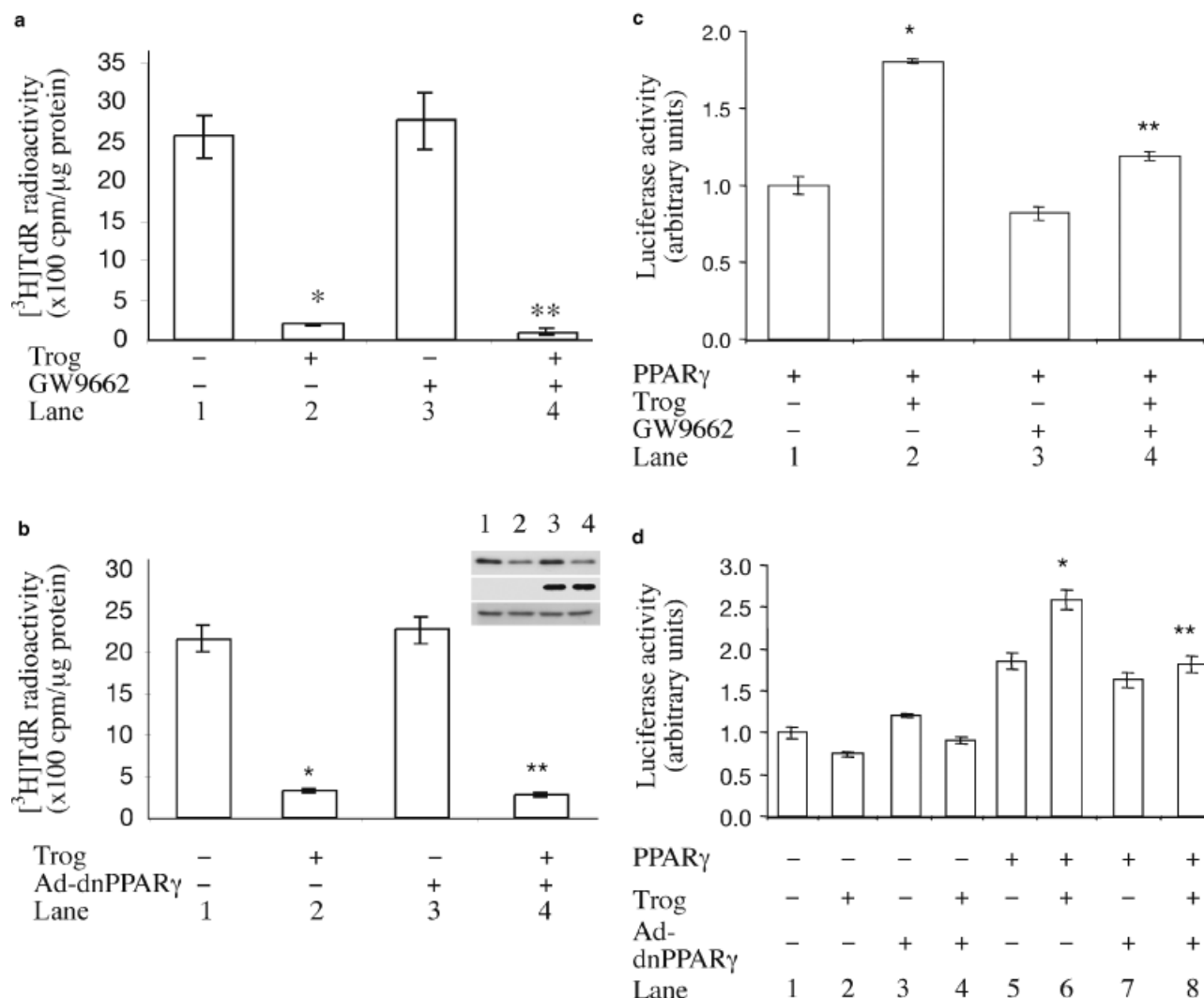
may also reflect the limited availability of other factors for PPAR $\gamma$  function in these cells such as retinoid X receptor or PPAR $\gamma$  co-activators. Similar results were obtained in primary keratinocytes (data not shown). The above findings prompted us to examine the expression of PPAR $\gamma$  in keratinocytes. Northern blot analysis with a mouse PPAR $\gamma$  cDNA probe showed that PPAR $\gamma$  mRNA was not detectable in C50 cells or in primary keratinocytes, and a strong band at 2.1 kb (PPAR $\gamma$ ) was detected in the control RNA of mouse fat tissue (Fig 7c). Western blot analysis repeatedly showed

**Figure 7**

**Absence of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) function in keratinocytes.** (a) C50 cells were transiently transfected in triplicate with Gal4-PPAR $\gamma$ -LBD and MH-100-TK-LUC and treated with troglitazone (Trog) (0.2–5  $\mu$ M), or 10  $\mu$ M Wy-14643 for 24 h. (b) C50 cells were transiently transfected in triplicate with a (PPAR response element (PPRE))<sub>3</sub>-TK-LUC construct along with an empty pcDNA3 vector (–) or pcDNA3-PPAR $\gamma$  (PPAR $\gamma$ ). Cells were treated with 5  $\mu$ M Trog (Trog) for 24 h. Luciferase activity was measured and normalized to an internal control ( $\beta$ -galactosidase). Inset of (b) shows the inhibition of 5  $\mu$ M Trog on expression of  $\beta$ -galactosidase, which is the combination of five independent experiments. Data are presented as mean  $\pm$  SD. \* $p < 0.05$  versus vehicle control. (c) Northern blot analysis was performed to determine PPAR $\gamma$  mRNA expression in primary keratinocytes (PK) and in C50 cells. Total RNA from mouse fat tissue was used as a positive control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as a loading control. (d) Western blot analysis with 30  $\mu$ g protein was performed for PPAR $\gamma$  expression in PK and in C50 cells. Ten micrograms protein from differentiated preadipocyte cell lysate (Upstate, Waltham, Massachusetts) was included as a positive control for PPAR $\gamma$  expression. A set of representative data from at least three independent experiments is presented.

that PPAR $\gamma$  protein levels in these cells were below the limit of detection, whereas PPAR $\gamma$  protein was readily detectable in differentiated preadipocytes even when 3-fold less protein was loaded (Fig 7d). The two bands in the preadipocyte sample were PPAR $\gamma$ 1 and PPAR $\gamma$ 2, respectively, as verified by using PPAR $\gamma$ 1 or PPAR $\gamma$ 2-transfected 293 cell lysates (data not shown). Taken together, these data suggest that PPAR $\gamma$  is not present at functional levels in cultured keratinocytes. Thus, the inhibitory effect of troglitazone on cell proliferation or cyclin D1 expression is likely to be PPAR $\gamma$ -independent.



**Figure 8**

**Effects of GW9662 and a dn-PPAR $\gamma$  (peroxisome proliferator-activated receptor  $\gamma$ ) on troglitazone (Trog) action in C50 cells.** (a) C50 cells in triplicate were pre-treated with 1  $\mu$ M GW9662 for 15 min, or (b) were pre-infected with an adenovirus expressing a dominant negative PPAR $\gamma$  (Ad-dnPPAR $\gamma$ ) or a control adenovirus (-) for 12 h. Cells were then treated with vehicle (-) or 5  $\mu$ M Trog (Trog) for an additional 24 h. Cells were pulsed with 1  $\mu$ Ci per mL of [<sup>3</sup>H]thymidine for 2 h before harvest. The [<sup>3</sup>H]thymidine ([<sup>3</sup>H]TdR) incorporated by cells was measured and normalized to protein concentration. (b) Inset: Cells were examined for cyclin D1 (upper band) and PPAR $\gamma$  (middle band) expression by western blot analysis.  $\beta$ -actin (lower band) was used as a loading control. (c) and (d) C50 cells were transiently transfected in triplicate with (PPAR response element (PPRE))<sub>3</sub>-TK-LUC construct along with a pcDNA3-PPAR $\gamma$  expression vector (PPAR $\gamma$ ) or a pcDNA empty vector (-). Sixteen hours later, cells were treated with 1  $\mu$ M GW9662 for 15 min (c) or infected with an adenovirus expressing a dominant negative PPAR $\gamma$  (Ad-dnPPAR $\gamma$ ) or a control adenovirus (-) for 12 h (d). Cells were treated with vehicle (-) or 5  $\mu$ M Trog (Trog+) for an additional 24 h. Luciferase activity was measured and normalized to an internal control ( $\beta$ -galactosidase). Data are presented as mean  $\pm$  SD. \* $p$  < 0.01 versus lane 1 in (a)-(c);  $p$  < 0.01 versus lane 5 in (d). \*\* $p$  > 0.05 versus lane 2 in (a) and (b);  $p$  < 0.01 versus lane 2 in (c) and lane 6 in (d). A set of representative data from at least two independent experiments is presented.

Consistent with the above conclusion, pre-treatment of C50 cells with 1  $\mu$ M GW9662, a potent PPAR $\gamma$  antagonist, failed to prevent the inhibitory effect of troglitazone on cell proliferation (Fig 8a). On the other hand, GW9662 at the same concentration effectively inhibited PPRE activity in these cells in the presence of both troglitazone and exogenous PPAR $\gamma$  (cf. lane 2 vs 4 in Fig 8c), indicating that GW9662 was working appropriately as a PPAR $\gamma$  antagonist at this concentration. To avoid the issue of possible non-specificity of PPAR $\gamma$  antagonists, a dnPPAR $\gamma$  was introduced into C50 cells by an adenovirus expression system. A 100% infection was achieved as monitored by green fluorescent protein (GFP) expression. The dnPPAR $\gamma$  did not alter the inhibitory effect of troglitazone on cell proliferation

or cyclin D1 expression (Fig 8b), although it did inhibit PPAR $\gamma$ -mediated induction of PPRE activity by troglitazone (cf. lane 6 vs 8 in Fig 8d).

## Discussion

A number of studies have demonstrated that activation of PPAR $\gamma$  inhibits cell growth and induces cell cycle arrest (reviewed in Fajas *et al*, 2001). In their pioneer study, Altioik *et al* (1997) showed that the TZD drug pioglitazone induced growth arrest and adipose differentiation of PPAR $\gamma$ -deficient NIH-3T3 cells only when exogenous PPAR $\gamma$  was introduced. They found that activation of PPAR $\gamma$  downregulated protein

phosphatase PP2A and thus inhibited the transcriptional activity of the E2F/DP complex due to an increase in the phosphorylation of these proteins. The inhibitory effect of PPAR $\gamma$  ligands on cell growth can also be the result of transcriptional upregulation of PTEN, a tumor suppressor gene, by PPAR $\gamma$  (Patel *et al*, 2001). PPAR $\gamma$  induces PTEN transcription by binding to two PPAR response elements in the sequence upstream of the PTEN gene (Patel *et al*, 2001). On the other hand, PPAR $\gamma$  is not always required for some PPAR $\gamma$  ligands to induce cell growth arrest. For example, TZD inhibit cell growth equally efficiently in PPAR $\gamma$  +/+ and PPAR $\gamma$  -/- mouse embryonic stem cells (Palakurthi *et al*, 2001). These studies clearly suggest that TZD may inhibit cell growth through PPAR $\gamma$ -dependent and/or PPAR $\gamma$ -independent mechanisms in a given cell context.

Our results suggest that troglitazone affects proliferation of keratinocytes through a PPAR $\gamma$ -independent pathway. The fundamental evidence for this is that troglitazone could not elicit PPRE activity in the cells unless exogenous PPAR $\gamma$  was introduced. In addition, neither PPAR $\gamma$  antagonists nor ectopic expression of a dominant negative PPAR $\gamma$  counteracted the inhibitory effect of troglitazone on cell proliferation. Although we do not exclude the possibility that PPAR $\gamma$  function is turned off through phosphorylation of PPAR $\gamma$  by MAP kinase (Hu *et al*, 1996), we believe that PPAR $\gamma$  is not present at functional levels in C50 cells or primary keratinocytes. We showed that PPAR $\gamma$  levels in these cells are below the limit of detection by Northern or Western blot analysis. This is, however, not completely in agreement with the observation that normal mouse skin expresses a low level of PPAR $\gamma$ , especially in the epidermis of newborn pups (Thuillier *et al*, 2000; Michalik *et al*, 2001). Interestingly, PPAR $\gamma$  expression in human skin was found to be confined to the suprabasal layers and its expression in cultured human keratinocytes was also barely detectable (Westergaard *et al*, 2001). These findings suggest that PPAR $\gamma$  expression in human skin keratinocytes is associated with keratinocyte differentiation (Westergaard *et al*, 2001). The lack of functional levels of PPAR $\gamma$  in cultured mouse skin keratinocytes may be because the cultured keratinocytes are undifferentiated.

The pathway by which PPAR $\gamma$  ligands induce cell cycle arrest is under intense study. Cdk inhibitor p21 is known to be involved in PPAR $\gamma$ -induced adipose differentiation. Interestingly, p21 expression is also upregulated by troglitazone in several tumor cells (Koga *et al*, 2001; Kim *et al*, 2002). p21 causes G1 arrest through inhibiting phosphorylation of Rb by cdk and inhibiting G1-S transition. Consistent with this, Rb -/- mouse embryo fibroblasts are resistant to PPAR $\gamma$  ligand-induced G1 phase arrest (Fajas *et al*, 2003). Our data show that p21 expression is not altered for up to 24 h by troglitazone in our cells. We demonstrated, however, that troglitazone strongly suppressed mitogen-induced cyclin D1 expression in keratinocytes. Inhibition of D-type cyclins can also cause G1 arrest because phosphorylation of Rb by cdk4/6 at the G1-S transition depends on the availability of D-type cyclins. D-type cyclins play a critical role in growth factor signaling and cell cycling and are targets for genetic and epigenetic alterations that underlie the development of many human neoplasias. For example, cyclin D1 is commonly over-

expressed in many tumor types, and constitutive overexpression of cyclin D1 in mouse mammary glands leads to development of carcinomas (Wang *et al*, 1994). In addition, overexpression of cyclin D1 in keratinocytes has been shown to cause increased proliferation and to partially override anti-tumorigenic signals (Martinez *et al*, 2000). Thus, inhibition of cyclin D1 expression appears to be a possible mechanism underlying the anti-proliferative effect of troglitazone in keratinocytes. Inhibition of expression of cdk2/4 may also contribute to the anti-proliferative effect of TZD. Clearly the mechanisms remain unclear and require further study.

It has been reported that the PPAR $\gamma$  ligand 15-PGJ<sub>2</sub> inhibited cyclin D1 transcription by activating PPAR $\gamma$  in MCF-7 cells (Wang *et al*, 2001). Ligand-bound PPAR $\gamma$  competes for the limited amount of co-activator p300 that is required for c-fos-mediated cyclin D1 transcription (Wang *et al*, 2001). We demonstrated a similar inhibition of cyclin D1 transcription by troglitazone in keratinocytes. The mechanisms by which troglitazone inhibited cyclin D1 transcription in keratinocytes is not clear, but it is likely to be PPAR $\gamma$ -independent for the reasons described above. We demonstrated that alteration in cyclin D1 protein degradation was also involved in the regulation of cyclin D1 protein expression by troglitazone in keratinocytes. This is indicated by the ability of proteasome inhibitors to inhibit the effect of troglitazone on cyclin D1 expression. We verified this observation by showing that troglitazone increased the ubiquitination of cyclin D1 protein, a process that is required for cyclin D1 degradation through the proteasome pathway. Interestingly, the PPAR $\gamma$  ligands, ciglitazone, and 15-PGJ<sub>2</sub>, are also shown to enhance cyclin D1 protein degradation in MCF-7 cells (Qin *et al*, 2003). Further study is needed to clarify the underlying mechanism.

Cellular proliferation is stimulated by extracellular mitogens through receptor-mediated signaling pathways. D-type cyclins function as critical sensors of these signals. Their expression is triggered and maintained by mitogenic stimulation, regardless of the position of the cell in the cycle, and growth factor withdrawal leads to rapid cyclin D protein destruction and G1-phase arrest (Sherr, 1995). Our results show that troglitazone rapidly and effectively inhibited mitogen-induced cyclin D1 expression and caused a G1 phase arrest in keratinocytes. It caused no inhibition of basal expression of cyclin D1, thus it appears likely that troglitazone may somehow inhibit mitogen signaling in these cells. Indeed, PPAR $\gamma$  ligands have been found to affect the extracellular signal-regulated kinase (Erk) pathway. In human macrophages and endothelial cells the PPAR $\gamma$  ligand 15-PGJ<sub>2</sub> was found to block activation of Erk1/2 by lipopolysaccharides and tumor necrosis factor  $\alpha$  independent of PPAR $\gamma$  (Eligini *et al*, 2002). In most other cells, however, PPAR $\gamma$  ligands activate the Erk pathway (Huang *et al*, 2002; Kim *et al*, 2002; Lennon *et al*, 2002). How PPAR $\gamma$  ligands interact with the Erk pathway is not clear, but involvement of reactive oxygen species has been suggested (Lennon *et al*, 2002). Nevertheless, a critical question that remains unanswered is whether altered activation of the Erk pathway is associated with the growth arrest action of PPAR $\gamma$  ligands in those cells. In addition to their effect on the Erk signaling, PPAR $\gamma$  ligands are also able

to interfere with growth factor receptor activation (Pignatelli *et al*, 2001; Gardner *et al*, 2003). In one study, PPAR $\gamma$  ligand 15-PGJ<sub>2</sub> was found to inhibit ligand-induced ErbB-2 and ErbB-3 tyrosine phosphorylation and to abolish ligand-induced cell proliferation in MCF-7 cells (Pignatelli *et al*, 2001). Although we do not know exactly how troglitazone inhibits mitogen-induced cyclin D1 expression and cell proliferation in mouse skin keratinocytes, it is likely that growth factor signaling is impaired by troglitazone in these cells.

Whether the inhibition of total protein synthesis is a general toxic effect of troglitazone is uncertain. First, the concentration of troglitazone (5  $\mu$ M) used is low and there were no signs of cell stress and cell death when cells were treated with troglitazone at this concentration. Secondly, it has been shown that troglitazone inhibited translation initiation in mouse embryonic stem cells independently of PPAR $\gamma$  through partial depletion of intracellular calcium stores and the resulting activation of protein kinase R (Palakurthi *et al*, 2001). Furthermore, the inhibited protein synthesis may contribute only partially to troglitazone's effects on cell proliferation and cell cycle arrest. The stronger inhibition by troglitazone on cyclin D1 incorporation of [<sup>35</sup>S]methionine than on total protein synthesis could be the result of inhibited cyclin D1 transcription as well as the result of enhanced cyclin D1 protein degradation. Hence, it is likely that the observed effect of troglitazone on cell proliferation or cell cycle arrest in C50 is not entirely due to toxicity.

In the mouse skin two-stage carcinogenesis model, tumor promotion is a rate-limiting step for tumor formation. It is proposed that the increased cellular proliferation during the promotion stage leads to selective clonal expansion of initiated cells, which makes it possible for an initiated cell to subsequently accumulate enough genetic mutations required for cancer development. Our data clearly show that both troglitazone and ciglitazone inhibit cellular proliferation of normal primary keratinocytes and initiated keratinocytes (C50 cells) *in vitro*. Given the important role of keratinocyte proliferation in mouse skin carcinogenesis, our data suggest that TZD may have utility in tumor prevention in mouse skin.

## Materials and Methods

**Cell culture** Primary keratinocytes were prepared as described previously (Thuillier *et al*, 2000). Briefly, 1–2-d-old SSIN newborns were euthanized. The epidermal keratinocytes were harvested by trypsinization, pooled and plated in enriched Waymouth's medium containing 1.2 mM calcium and 10% fetal bovine serum. The cells were allowed to attach at 37°C in 5% CO<sub>2</sub> for 2.5 h, and medium was then replaced with serum-free KGM-2 (Biowhittaker, Walkersville, Maryland) containing 0.03 mM calcium. C50 cells, a non-tumorigenic keratinocyte cell line (a gift from Dr A.J.P. Klein-Szanto, Fox Chase Cancer Center, Philadelphia, Pennsylvania) derived from spontaneously immortalized normal mouse skin keratinocytes (Ruggeri *et al*, 1991), were maintained and plated at 37°C in 5% CO<sub>2</sub> in Eagle's Minimal Essential Medium (EMEM) supplemented with 1% fetal bovine serum and a variety of growth factors as described (Dotto *et al*, 1988). The EMEM medium was replaced with KGM-2 medium containing 0.03 mM of calcium 16 h after plating. Both primary keratinocytes and C50 cells were plated at a density such that they would become about 80% confluent by the

third day of growing in KGM-2 without changing medium (except where otherwise described). The cells were then stimulated with pre-warmed, fresh KGM-2 medium containing vehicle or appropriate treatment. All animal experiments were approved by the institutional animal use committee at the University of Texas, MD Anderson Cancer Center and were performed in accordance with the principles and procedures outlined in the NIH guidelines for the care and use of experimental animal.

**Chemicals** PPAR $\gamma$  ligands, troglitazone, and ciglitazone, were gifts from Parke-Davis Pharmaceutical Research Division of Warner-Lambert Company (Ann Arbor, Michigan). PPAR $\alpha$  ligand 4-chloro-6-(2,3-xylyldino)-2-pyrimidinylthioacetic acid (Wy-14643) was purchased from Chemsyn Science Laboratories (Lenexa, Kansas). Proteasome inhibitors Alln and MG-132 were purchased from CalBioChem (La Jolla, California). PPAR $\gamma$  antagonist GW-9662 was purchased from Cayman Chemical (Ann Arbor, Michigan). All chemicals were dissolved in dimethyl sulfoxide.

**[<sup>3</sup>H]Thymidine incorporation assay** Primary keratinocytes or C50 cells grown to 80% confluence in six-well plates were treated in triplicate with vehicle or PPAR $\gamma$  ligands for 3–24 h. Two hours prior to harvest, cells were pulsed with 1  $\mu$ Ci per mL of [<sup>3</sup>H-methyl]thymidine ([<sup>3</sup>H]thymidine) (CalBioChem, UK). The medium was removed and cells were rinsed twice with ice-cold phosphate-buffered saline. Cells were then incubated on ice with ice-cold 10% (w/v) trichloroacetic acid for 10 min once and 5 min twice to fix cells and precipitate DNA. The cells were lysed with 1 mL per well of lysis buffer containing 0.3 N NaOH, 1% sodium dodecyl sulfate (SDS) for 15 min at room temperature. Cell lysates were transferred to scintillation vials containing 5 mL scintillation liquid and mixed well. [<sup>3</sup>H]thymidine radioactivity was measured and the values were normalized to protein concentration.

**Flow cytometry** C50 cells at 80% confluence in 100 mm dishes were treated with PPAR $\gamma$  ligands for 24 h and harvested by trypsinization. Single cell suspensions were obtained by filtering the cells through a 70  $\mu$ m mesh once. Cells were fixed on ice in ice-cold 70% ethanol for 1 h and stained with 0.5 mL propidium iodide phosphate-buffered saline solution (40  $\mu$ g per mL). The stained cells were diluted with propidium iodide solution to about 5  $\times$  10<sup>5</sup> cells per mL and analyzed on a Coulter EPICS Elite flow cytometer (Coulter, Clarkson, Michigan) using a 610 nm bypass filter and aggregate discrimination. Cell cycle analysis was carried out using Multicycle DNA cell cycle analysis software from Phoenix Flow systems (San Diego, California).

**Western blot analysis** Western blot analysis was performed as previously described (Thuillier *et al*, 2000). Thirty micrograms of whole cell lysate of each sample was heated at 95°C for 10 min, denatured and fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The blots were blocked for 1 h in Tris-buffered saline containing 5% non-fat milk and 0.1% Tween 20. They were immunostained at room temperature for an additional 1 h with antibodies against cyclin D1 (C-20), cyclin A, cyclin B1, cyclin E, cdk2, cdk4, p21, or PPAR $\gamma$  (E-8) (Santa Cruz Biotechnology, Santa Cruz, California). The blots were washed extensively with Tris-buffered saline with 0.1% Tween-20. Bound primary antibody was detected with horseradish peroxidase-conjugated second antibody and chemiluminescent substrate (Perkin-Elmer Life Science, Boston, Massachusetts).  $\beta$ -actin was used as a loading control.

**[<sup>35</sup>S]methionine incorporation assay** C50 cells in triplicate were treated with vehicle or 5  $\mu$ M troglitazone for 5 h and pulsed with 200  $\mu$ Ci per mL [<sup>35</sup>S]methionine (ICN, Costa Mesa, California) in methionine-free EMEM medium for an additional 1 h. Cells were washed twice with ice-cold phosphate-buffered saline and harvested in radioimmunoprecipitation assay buffer. Whole-cell lysates were obtained by centrifugation for 15 min at 13,000 g. Protein concentration was determined with the BCA kit (Bio-Rad,



Richmond, California). Three hundred micrograms of total protein was each used for measurement of [<sup>35</sup>S]methionine radioactivity and for cyclin D1 immunoprecipitation. Cyclin D1 protein immunoprecipitation was performed as described in the following section "detection of cyclin D1 protein degradation" and fractionated by 10% SDS-polyacrylamide gel electrophoresis. The gel was dried and exposed to an X-ray film.

**Northern blot analysis** Total RNA was extracted with Tri-Reagent (MRC, Cincinnati, Ohio) following the manufacturer's protocol. Ten micrograms of total RNA of each sample was denatured and separated on 1% agarose/6% formaldehyde gel, and then transferred to nylon membranes. A [<sup>32</sup>P]dCTP-labeled cDNA probe of cyclin D1 or PPAR $\gamma$  was hybridized to the blots at 65°C for 2 h. The blots were then washed twice each for 15 min in 0.1% SDS/2  $\times$  NaCl/sodium citrate solution (where 1x is 0.15 M NaCl/15 mM sodium citrate) at room temperature and once for 30 min with 0.1% SDS/0.1  $\times$  NaCl/sodium citrate solution at 60°C and exposed to X-ray film at -80°C. The blots were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA as a loading control.

**Detection of cyclin D1 protein degradation** To determine if troglitazone promotes cyclin D1 protein degradation through the proteasome pathway, C50 cells at about 80% confluence were pre-treated with proteasome inhibitor Alln or MG-132 for 15 min before application of troglitazone. Whole cell protein was extracted in radioimmunoprecipitation assay buffer. Cyclin D1 levels were measured by western blot analysis. To determine ubiquitination of cyclin D1 protein, C50 cells were treated with vehicle, or 5  $\mu$ M troglitazone for 6 h and whole cell protein was extracted. Three hundred  $\mu$ g of total protein was incubated with 10  $\mu$ g cyclin D1 antibody (Santa Cruz Biotechnology) at 4°C overnight. Protein A agarose beads were then directly added to each sample and incubated at 4°C for an additional 1 h. The beads were washed four times with radioimmunoprecipitation assay buffer by short spin and immunoprecipitates were eluted from the beads with 30  $\mu$ L of 1  $\times$  SDS sample buffer. The eluted proteins were heated at 95°C for 10 min, processed for Western blot analysis and immunostained with an anti-ubiquitin antibody (P4D1, Santa Cruz Biotechnology). The blot was stripped and reprobed with cyclin D1 antibody as an immunoprecipitation control. As a loading control, thirty  $\mu$ g of total protein of each sample was loaded along with the immunoprecipitation sample and transferred to the same blot and probed with anti- $\beta$ -actin antibody (Santa Cruz Biotechnology).

**Transient transfection** Primary keratinocytes (1  $\times$  10<sup>6</sup> per well) or C50 cells (4  $\times$  10<sup>5</sup> per well) were plated in six-well plates and grown to about 60% confluence the next day. Following replacement of medium with fresh KGM-2, cells were transfected with plasmid(s) as indicated below for 16 h using Eugene 6 (Roche Diagnostics, Indianapolis, Indiana) with a DNA: Eugene 6 ratio of 1  $\mu$ g:3  $\mu$ L. To test the effect of troglitazone on cyclin D1 promoter activity, each well was transfected with 1  $\mu$ g of a luciferase reporter construct driven by a 1745 bp cyclin D1 promoter (a kind gift from Dr Chris Albanese, The Albert Einstein Cancer Center, New York, New York). To test for the ability of troglitazone to bind to PPAR $\gamma$  in C50 cells, cells were co-transfected with 1  $\mu$ g per well Gal4-PPAR $\gamma$ -LBD and 1  $\mu$ g per well MH-100-TK-LUC (a generous gift from Dr R.M. Evans, The Salk Institute, San Diego, California). Gal4-PPAR $\gamma$ -LBD is a chimeric receptor in which the ligand-binding domain (LBD) of mouse PPAR $\gamma$  is fused to the DNA-binding domain of the yeast transcription factor Gal4. The chimeric receptor is able to bind to the Gal4 DNA binding motif but activates transcription only in response to ligands bound to the LBD of PPAR $\gamma$  (Nagy *et al*, 1998). To test for the ability of troglitazone to activate PPARE activity, cells were co-transfected with 1  $\mu$ g per well of a PPARE luciferase reporter construct (PPRE)<sub>3</sub>-TK-LUC (generously provided by Dr R.M. Evans), along with 1  $\mu$ g per well of a pcDNA3-PPAR $\gamma$  expressing vector (a kind gift from Dr

V.K. Chatterjee, University of Cambridge, UK) or 1  $\mu$ g per well of the empty pcDNA3 vector (Invitrogen, Carlsbad, California). As an internal control, 0.125  $\mu$ g per well of pCMV- $\beta$ -galactosidase plasmid was always co-transfected. After 16 h, cells were given fresh pre-warmed KGM-2 medium and treated with vehicle or troglitazone or other PPAR ligands for an additional 24 h. Protein was extracted in luciferase lysis buffer (Tropix, Bedford, Massachusetts). Luciferase activity was measured by a luminometer (Tropix) and normalized to the  $\beta$ -galactosidase activity.

**Generation and use of adenovirus expressing a dominant negative PPAR $\gamma$**  The flag epitope-tagged human dominant negative PPAR $\gamma$  (dnPPAR $\gamma$ ) cDNA was obtained by cutting the pcDNA-dnPPAR $\gamma$  (Gurnell *et al*, 2000) (a kind gift from Dr V.K. Chatterjee, University of Cambridge, Cambridge, UK) with KpnI/XbaI. The cDNA was put into a pAd-track vector at the KpnI/XbaI sites and then cloned into pAdEasy-1 adenovirus vector by homologous recombination according to the manual supplied with the AdEasy XL adenoviral vector system (Stratagene, La Jolla, California). The empty pAd-track vector was also cloned into pAdEasy-1 as a control. The adenovirus pAd-track-dnPPAR $\gamma$  and pAd-track, which express the dnPPAR $\gamma$  plus GFP and control GFP alone, respectively, were produced and amplified in Ad-293 cells and purified by CsCl gradient centrifugation. In the experiments where adenovirus vectors were used, cells were pre-incubated with pAd-track-dnPPAR $\gamma$  and pAd-track-GFP at a multiplicity of infection of 1:20 for 12 h and troglitazone was directly added into the medium.

**Statistics** Data obtained from the [<sup>3</sup>H]thymidine incorporation assays, [<sup>35</sup>S]methionine incorporation assay and from the transient transfection assays were analyzed with one-way ANOVA to compare means between the vehicle control and treatment groups using SPSS 10 (SPSS Mac V.10, SPSS, Chicago, Illinois).

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Address correspondence to: Susan M. Fischer, The University of Texas M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX 78957, USA. Email: smfischer@mdanderson.org

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